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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This project is aimed at studying specific Rho signal transduction pathways in normal and breast carcinoma cell lines. The members of Rho family of Ras-related proteins are critical downstream components of signaling pathways. The Rho GTPases are involved in a variety of cellular functions. The Rho GTPases are active when bound to GTP and switch to inactive form when the protein bounf GTP is hydrolyzed. Rho GTPase activating protein (Rho GAP) converts active Rho into inactive Rho. We have cloned a Rho GAP encoded by the human chromosome 13q12. We have characterized the biochemical activity of the bacterially expressed Rho GAP by investigating its effect on native GTPase activity of Rho protein. The physiological role of Rho GAP was established by transfecting cells with Rho GAP construct and showing its effect on actin reorganization. Furthermore, constructs containing altered sequences of Rho GAP cDNAs were made to investigate their effect on cellular phenotypes of normal and breast carcinoma cell lines.

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FOREWORD

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Gampal 8/20/01

PI - Signature Date

INTRODUCTION

Change of Grantee Institution: The P.I. initiated this research at the Temple University School of Medicine. The specific tasks proposed for the first year were carried out at Temple University School of Medicine. The P.I. moved to Fordham University in Fall 2000. The approval for the transfer of grant from Temple University to Fordham University was sought from the US Army command, and it took a few months to complete all formalities for transfer of the grant. It took the P.I. sometime to set up his new laboratory. We had, however, continued to perform the specific tasks as proposed in the original application. The results presented here describe a summary of our progress to date.

Background:
The development of multicellular organisms is mediated by a highly complex and coordinated signaling network. The aberrant expression and/or function of various members of signaling pathways has been conclusively linked to developmental abnormalities and a variety of chronic diseases including cancer. This proposal addresses downstream elements of Ras signal transduction pathway that may mediate some aspects of malignant transformation. Ras- and Ras-related families, Rab, Rac and Rho are small GTP binding proteins. These proteins are key components of signal transduction pathways that link extracellular proliferation and/or differentiation signals such as growth factors and oncogenes to nuclear transcription of specific genes which promote these processes (1-3). Indeed, mutations affecting Ras and/or Ras pathway are frequently seen in human cancers including lung, colon, endometrium, ovary, pancreas, thyroid and a smaller proportion of breast cancers (4). It has been demonstrated in several cancers where Ras is not mutated that downstream effectors of Ras signaling pathway are involved in tumorigenesis. We have shown aberrant expression of Rho GAP, a downstream member of the Ras-Rho signaling cascade, in breast tumorigenesis.

The Rho family of small GTP-binding proteins which include Rho, Rac, and cdc42 belong to the Ras superfamily (2), and comprises several known proteins . These proteins bind GTP and hydrolyze it via low levels of intrinsic GTPase activity. They function as molecular switches in the transduction of signal generated by the activation of a variety of cell surface receptors including the EGF receptor, the erB-2 gene product, the CSF-1 receptor, and the cell adhesion receptor integrins (2,3). When cells receive an appropriate stimulus, these GTPases are converted to the active GTP-bound state by activated guanine nucleotide exchange factors (GEFs), whereas the GTP-bound form is later rendered inactive by the GTPase-activating proteins (GAPs) (5). The activated Rho GTPases interact with multiple downstream effector targets which serve to transmit the incoming signals from the small G-proteins leading to cellular responses such as actin-cytoskeleton changes (3), cell polarization (2), transcriptional activation (6-8), focal adhesion formation (9), and stimulation of DNA synthesis (10). Rho is required for growth factor induced formation of stress fibers and focal adhesions (11), regulation of cell morphology (12), cell aggregation (13), cell motility (14), and cytokinesis (15).

The activity of Rho is negatively regulated by GTPase activating proteins (GAPs). Several Rho GAPs have been identified, and some of the GAPs have been shown to stimulate specific Rho family proteins preferentially (16). Expression of GAP domain of p190 in Swiss 3T3 cells has been shown to cause changes in morphology and lysophosphatidic acid (LPA)- and serum- induced stress fiber formation (11). An aberrant expression, regulation and/or activity of the proteins involved in cytoskeletal organization may lead to transformation of a normal cell, and alter the invasiveness and metastatic potential of a malignant cell.

The best characterized function of Rho family proteins involves their regulation of specific filamentous F-actin organization. The three major cytoskeletal protein networks, namely, actin filaments, microtubules, and intermediate filaments are involved in a variety of processes. Specifically, actin cytoskeleton is a highly dynamic structure responsive to diverse extracellular signals. Lamellopedia, membrane ruffles, actin stress fibers, focal adhesions, and filopodia are representative distinct actin-based structures, which are regulated by specific Rho family proteins in fibroblasts and other cell types (17). Because an altered morphological phenotype is a key feature of transformed cells, the Rho mediated pathways that control cytoskeleton reorganization may contribute to the general phenomena of transformation and invasiveness. The ability of a eukaryotic cell to maintain or change its shape and its degree of attachment to substratum in response to extracellular signals is largely dependent on the rearrangement of the actin cytoskeleton. Cytoskeletal rearrangements play a crucial role in processes such as cell motility, cytokinesis, cell growth, and contact inhibition- and alteration in these processes is a hallmark of tumor cells.

Body

We had originally proposed to characterize a human Rho GTPase Activating Protein (GAP) that we had cloned from human chromosome 13q21. The specific aims proposed in the original application are stated below.

Specific aim 1: a) investigate the expression profile and level of Rho GAP expression in breast cancer cell lines and correlate lack of its activity to gene mutation, and b) express the novel Rho GAP, and determine its biochemical and functional properties such as GAP activity, substrate specificity and phospholipase C-delta stimulation.

Specific aim 2: Investigate the role of Rho GAP in normal breast cells using antisense constructs to sequester the native Rho GAP message and determine its effect on lysophosphatidic acid induced Rho-mediated actin reorganization and phenotypic changes in normal and cancer breast cells.

Specific aim 3: Use the yeast two hybrid system to identify other proteins that interact with the novel Rho GAP and are involved in Rho-mediated signaling.

The specific tasks proposed for year 2 are described below.

Task 5: **Months 13-16**: Determination of enzymatic activity and substrate specificity.
Task 6: **Months 17-19**: Cloning of Rho GAP and its mutants in sense and antisense orientation.
Task 7: **Months 20-23**: Cytoskeleton reorganization in transfected cells.

The following section describes the progress made in specific tasks assigned for the second year of the grant.

Task 5:
The entire coding sequence of Rho GAP was cloned in a bactrerial expression vector pGEX at sites SmaI and SalI. The cloning strategy consisted of the following steps, A pair of PCR primers was synthesized from the 5' and 3' ends. The primers contained a SmaI restriction sequence in the 5' primer and a SalI restriction sequence in the the 3'primer. The primer

sequences are presented in Figure 1. The Rho GAP coding sequence was amplified from a construct that we have made in pBKCMV mammalian expression vector. A 'hot PCR' protocol was followed by denaturing the template at 94^0 C and adding the enzyme at 72^0 C. These conditions eliminate any nonspecific amplification of the template. The cycling conditions were as follows. Denaturation @94^0 C for 15 sec, annealing at 62^0 C for 30 sec and extension at 68^0 C for 4 min for 20 cycles, followed by a 7 min extension at 72^0 C. A high fidelity Taq polymerase was used for these purposes. A clean amplification product was obtained under these conditions. The PCR amplified product was subsequently digested with Sma I and Sal I restrcition endonucleases. The double digested product was purified on an agarose gel, extracted with phenol/chlolroform and precipitated with ethanol. The purified fragment was quantited by running on a gel along with known amounts of DNA. Likewise, approximately 1 μ g of pGEX vector was digested with Sma I and Sal I restriction enzymes and dephosphorylated with calf intestinal alkaline phosphatase. The dephosphorylated vector (20 ng) was ligated to 100 ng of Rho GAP fragment in a 10 μ l reaction mixture. The ligated mixture was used to transform DH5α competent cells. The recombinant clones were picked up, grown in ampicilin containing medium, and processed for preparing plasmid DNA.

Evaluation of cloned construct:
The expression construct was verified by restriction digestion of the pGEX DNA. The size of insert and presence of specific sites confirmed the presence of desired fragment in the vector cloned in appropriate orientation. However, these tests did not confirm if the insert was in the right reading frame and there were no internal point mutations in the insert. In order to ascertain that there were no sequence mutations in the construct, five independent clones were subjected to a one pass sequencing. The sequence revealed that the cDNA was in frame with the plasmid open reading frame, and was predicted to translate the expected protein with a leader sequence supplied in the vector. With these analyses the construct was used for expression in the bacterial host after appropriate induction.

Small scale induction of recombinant pGEX Rho GAP: A 2ml culture of in LB with 100 μ g per ml of ampicillin was grown overnight. A 10 ml culture was subsequently grown with the 0.1 ml inoculum of overnight culture until an OD of 0.5-1.0 was reached at 590 nm wavelength. 1 ml of the above culture was removed to serve as an uninduced culture. The remainder was further incubated with IPTG (0.6 mM final concentration) and grown for 3-5 hours. The sample aliquots were spun down after measuring OD, suspended in PBS, boiled in the presence of 2X SDS sample buffer. The protein samples were analyzed by SDS-polyacrylamide gel electrophoresis. Expression of GST fusion protein was monitotred by using GST detection kit. The results showed specific induction of the desired protein.

Large scale induction: A 5 ml overnight culture was used to inoculate 500 ml of LB medium containing 100 μ l ampicillin per ml. An aliquot was removed after the OD has reached ~1.0 and saved as an uninduced control. The remainder was then induced with IPTG and grown for an additional 3h as above. The culture was centrifuged and the cells resuspended in four volumes of lysis buffer. The cells were completely lysed and debris removed by centrifugation. The crude extract was used for affinity purification as described below.

Affinity purification: The recombinant protein was translated in frame with GST protein that was part of the vector. The GST was exploited to purify the protein by using glutathione sepharose 4B columns. The crude supernatant was selectively retained on and subsequently eluted from a regenerated glutathioneSepharose 4B column as per the manufacturer's

instructions. A nearly homogeneous mixture of desired protein was obtained. The protein as purified above was obtained as GST fusion which is cleavable by factor Xa.

Determination of GAP activity of recombinant Rho GAP: The activity of purified Rho GAP was checked on Rho A GTPase as follows. The GTPase assay was performed according to the method of Self and Hall (18). Fifty ng each of Rho A protein was preloaded with [gamma-^{32}P] GTP (6000 Ci/mmol, 10μCi/μl) in 20 μl of 20 mM Tris-HCl, pH 7.6, 0.1 mM DTT, 25 mM NaI, and 4 mM EDTA for 10 min at 30°C. The mixture was placed on ice and MgCl2 added to a final concentration of 17 mM. Three μl of the preloaded protein was diluted with buffer (20 mM Tris-HCl, pH 7.6, 0.1 mM DTT, 1 mM GTP, 1 mg/ml Bovine Serum albumin) to give a final volume of 30 μl. A 5 μl sample was removed (time zero) and diluted into 1 ml cold assay buffer (50 mM Tris HCl, pH 7.6, 50 mM NaCl and 5 mM MgCl2). For GAP stimulated GTPase assays, an aliquot of GAP proteins was added at this stage. The remainder of the reaction was incubated at 20°. Samples (5 μl) were removed at 5, 10, 15 min. intervals and diluted with cold assay buffer (1 ml). The reaction mixture was filtered through pre wetted nitrocellulose filters, and the amount of radioactivity remaining bound to the protein was determined by scintillation counting. The result was expressed in terms of % bound GTP remaining vs time. As shown in Table 1 these assays confirm the status of the novel protein as a GAP for Rho. The bacterially purified protein appeared suitable to establish its activity.

The GTPase stimulatory activity of Rho GAP was predicted based on the presence of GAP domain and GTP binding domain, and its striking similarity with rat protein. Our experimental observations confirm these predictions. The demonstration of PLC-delta activity will imply a functional association between the Rho and PIP2 systems, and this novel Rho GAP could be a key molecule for regulation of PIP2 hydrolysis by Rho in breast epithelium.

We now propose to investigate if this protein has any PLC-delta stimulation activity. Such studies have also been carried out successfully in vitro with purified GST fusion proteins isolated from bacterial extracts (19). While PLC-beta and PLC-gamma are regulated by heterotrimeric G-proteins, nothing is known about PLC-delta activation mechanism. PLC-delta stimulation by novel Rho GAP will be assayed by evaluating the increase in the rate of hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by using radiolabeled PIP2 at varying concentration of Ca++. Should there be problems with PLC-delta stimulation in vitro, we will resort to cotransfection of PLC-delta and Rho GAP constructs in vivo and using the extracts to score for increase in PIP2 hydrolysis over the background.

Task 6:

Preparation of mutant and antisense Rho GAP constructs: We have prepared two sets of constructs that contain altered Rho GAP. In one of these, we have deleted either the GAP domain alone or the entire C-terminal inclusive of the GAP domain. We believe the physiological effects of this protein are largely attributable to its specific Rho GAP activity. These constructs, as shown in Figure 2 were prepared in the following manner.

A. The middle region of the cDNA corresponding to amino acid residues 560-702 was selectively removed, so that it would translate a protein that does not contain the GAP domain.

B. The sequences down stream of amino acid residue 560 were completely removed, so that it translates only the amino half of the protein.

C. The entire construct was cloned in an anti sense orientation, so as to enable it to sequester any sense strand before it is translated.

All these constructs are proposed for transfection into normal and breast carcinoma cell lines to evaluate their effect on cell growth and morphology. It is proposed to assess their effect both in transient expression and stable expression.

Task 7:
Effect of Rho GAP expression on cell morphology: The best characterized effect of Rho proteins is on actin reorganization and cytoskeleton. The best characterized cell system for investigating these effects are fibroblast cell lines. We have, therefore, standardized our system on these cell lines before studying breast epithelial cell lines.

The effect of transfected gene(s) on cellular morphology: The Rho GAP-transfected and control (transfected with empty vector) fibroblast cells were grown in appropriate culture medium containing 0.11 g/L sodium pyruvate 4.5g/L glucose, 10% fetal calf serum at 37°C in a CO_2 incubator. Cells were seeded at a density of $3.7 \times 10^3/cm^2$ and passaged two times per week. For analysis of actin reorganization, cells were seeded at a density of $3\text{-}5 \times 10^4$ per well on 13 mm glass coverslips. The cells grown on coverslips were fixed for 20 minutes in 3% paraformaldehyde, washed to remove excess fixative, and then permeabilized in 0.2% Triton X-100. The coverslips were washed twice with PBS and then incubated with 200 µl of 1 µg/ml tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin for 30-40 minutes in the dark to localize actin filaments. Coverslips were washed extensively with PBS and mounted for viewing under a fluorescence microscope. It warrants mention that the actin cytoskeleton in epithelial cells is not as marked as fibroblast cells, we have initiated our studies on fibroblast cells to set the baseline parameters. As can be seen in figure 3 that polymerized actin organized as stress fibers in control cells, whereas stress fiber formation was inhibited in Rho GAP overexpressing cells. These initial studies demonstrate that the Rho GAP indeed influences actin reorganization and confirms the biochemical activity as established above with its physiological function. We are proceeding ahead by repeating the same experiments in normal breast cells and breast carcinoma cells.

Figure 1

A: Primer Sequences used to amplify the RhoGAP insert from a plasmid clone

I) 5' primer with restriction sites for SmaI and NotI

NotI

ACG TCC CGG GGC GGC CGC CGT TGA ACA AGT GTG CCT CA 3'

SmaI — Gene specific sequence

A. 3' Primer with restriction sites for KpnI and SalI

SalI

ACG TGG TAC CGT CGA CGT TTG ATG TCA CAC TGG G 3'

KpnI — Gene specific sequence

B: Map of bacterial expression vector pGEX

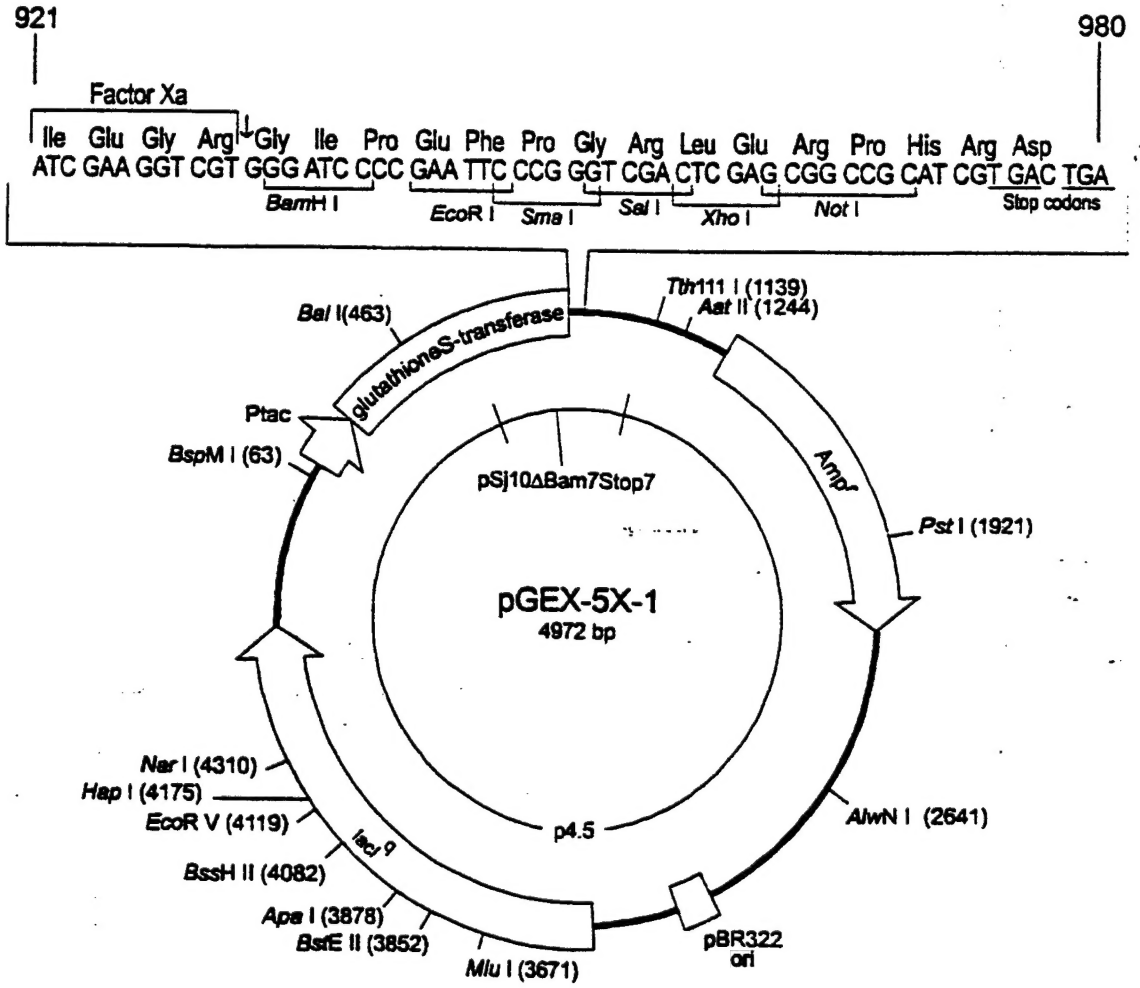
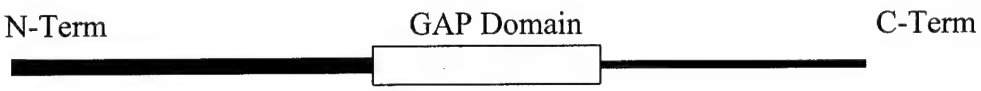
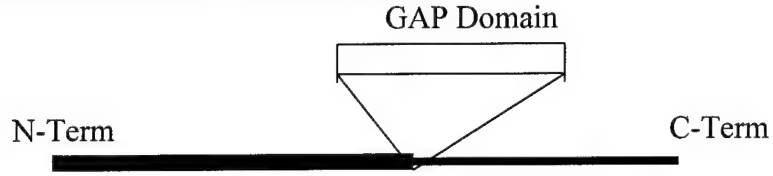


Figure 2: Rho GAP Constructs

A. Wild Type Construct



B. Construct with GAP domain deleted



C. Construct truncated at the GAP domain

D. Antisense construct

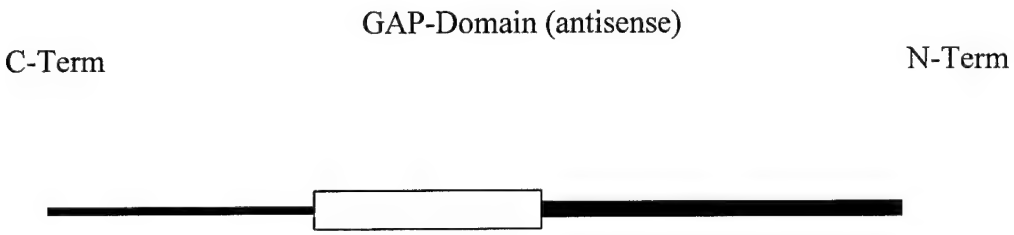


Figure 3: Effect of Rho GAP transfection on actin organization.

Fibroblast cell transfected with control vector

Fibroblast cell transfected with Rho GAP construct

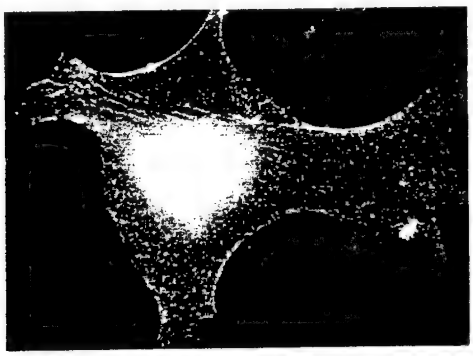


Table 1: GTPase Activation by Rho GAP

Time of Incubation	% Radioactive GTP remaining bound to Rho[@]
0 min	100
5 min	70± 7
10 min	55± 10
30 min	20±9

@ Approximately 50 ng of labeled GTP loaded Rho was used to evaluate its GTPase activity. The results are an average of three replicates.

Effect of Recombinant Rho GAP on Rho GTPase activity:

Time of Incubation	% Radioactive GTP remaining bound to Rho[@@]
0 min	100
5 min	45± 12
10 min	10± 8

@@ The labeled GTP loaded Rho (~50 ng) was mixed with 200 ng of recombinant Rho GAP. The GTPase stimulatory activity of GAP was determined by measuring the amount of labeled GTP remaining bound to Rho.

Key Research Accomplishments:

- We have bacterially expressed the chromosome 13q12 Rho GAP.
- The biochemical activity of Rho GAP was characterized.
- Several constructs of Rho GAP were made in an expression vector for transfection into normal breast cells and breast carcinoma cells.
- The physiological significance of Rho GAP was evaluated by transfecting Rho GAP construct into a fibroblast cell line.

Reportable Outcomes:
None.

Conclusions:
We had previously shown that expression of a novel transcript that had sequence similarity to rat Rho GAP sequence was altered in breast carcinoma cell lines. We believed that the putative Rho GAP is functionally active. Rho family of proteins shares homology with the Ras super family. Our working hypothesis was that breast carcinoma where Ras mutations have not been detected could still arise from aberrant Ras signaling by virtue of loss of activity of members of Rho family or factors/proteins that affect the activity of Rho proteins. We have bacterially expressed the putative Rho GAP and evaluated its GTPase stimulating activity in biochemical assays. To further associate the biochemical activity with its well-established physiological role we have shown that transfected Rho GAP can alter actin organization of fibroblast cell lines. We have also prepared a variety of Rho GAP constructs (sense, anti-sense, truncated etc) in an expression vector to transfect normal and breast carcinoma cell lines. We are optimistic that with these constructs we will be able to gather valuable information about potential role of the Rho GAP in breast malignancy.

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